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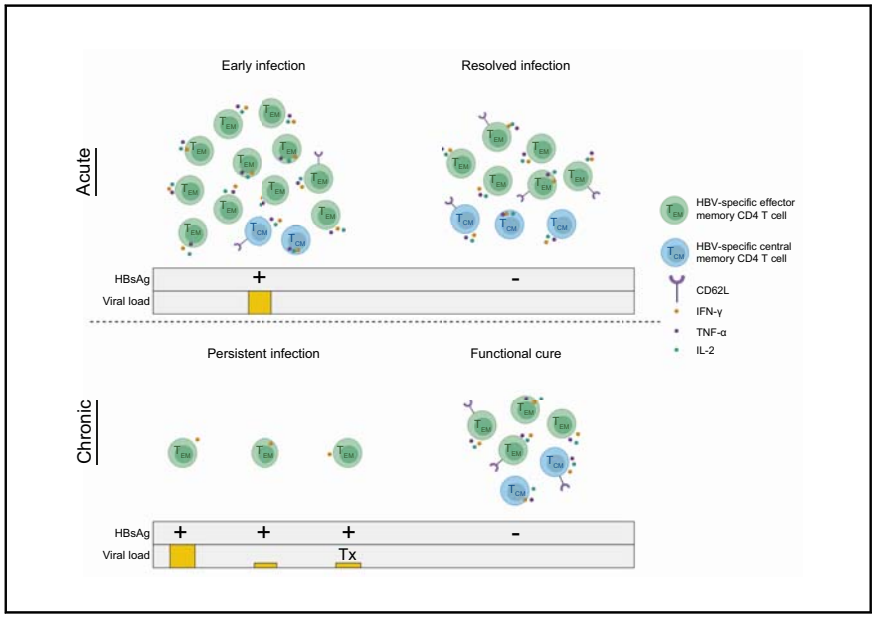
Hepatitis B virus-specific CD4 T cell responses differentiate functional cure from chronic surface antigen^{+} infection

Graphical abstract



Highlights

- HBV-specific CD4 T cells can be analysed directly *ex vivo*, without the need for *in vitro* expansion.
- Functional HBV-specific CD4 T cell responses are stronger in functional cure *vs.* chronic infection.
- MHC-class II multimers identify HBV-specific CD4 T cells reliably in HBsAg- patients, but rarely when HBsAg is present.
- CD4 T cell immunity should be considered as a target for HBV immunotherapies.

Authors

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Lay summary

Immunotherapy is a form of treatment that relies on harnessing the power of an individual's immune system to target a specific disease or pathogen. Such approaches are being developed for patients with chronic HBV infection, in an attempt to mimic the immune response in patients who control HBV infection spontaneously, achieving a so-called functional cure. However, what exactly defines protective immune responses remains unclear. Herein, we show that functional cure is associated with robust responses by HBV-specific CD4 T cells (a type of immune cell).

https://doi.org/10.1016/j.jhep.2022.05.041
 J. Hepatol. 2022, 77, 1276–1286

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Hepatitis B virus-specific CD4 T cell responses differentiate functional cure from chronic surface antigen^{+} infection

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Background & Aims: With or without antiviral treatment, few individuals achieve sustained functional cure of chronic hepatitis B virus (HBV) infection. A better definition of what mediates functional cure is essential for improving immunotherapeutic strategies. We aimed to compare HBV-specific T cell responses in patients with different degrees of viral control.

Methods: We obtained blood from 124 HBV-infected individuals, including those with acute self-limiting HBV infection, chronic infection, and chronic infection with functional cure. We screened for HBV-specific T cell specificities by ELISpot, assessed the function of HBV-specific T cells using intracellular cytokine staining, and characterized HBV-specific CD4 T cells using human leukocyte antigen (HLA) class II tetramer staining, all directly *ex vivo*.

Results: ELISpot screening readily identified HBV-specific CD4 and CD8 T cell responses in acute resolving infection compared with more limited reactivity in chronic infection. Applying more sensitive assays revealed higher frequencies of functional HBV-specific CD4 T cells, but not CD8 T cells, in functional cure compared to chronic infection. Function independent analysis using HLA multimers also identified more HBV-specific CD4 T cell responses in functional cure compared to chronic infection, with the emergence of CD4 T cell memory both after acute and chronic infection.

Conclusions: Functional cure is associated with higher frequencies of functional HBV-specific CD4 memory T cell responses. Thus, immunotherapeutic approaches designed to induce HBV functional cure should also aim to improve CD4 T cell responses.

Lay summary: Immunotherapy is a form of treatment that relies on harnessing the power of an individual's immune system to target a specific disease or pathogen. Such approaches are being developed for patients with chronic HBV infection, in an attempt to mimic the immune response in patients who control HBV infection spontaneously, achieving a so-called functional cure. However, what exactly defines protective immune responses remains unclear. Herein, we show that functional cure is associated with robust responses by HBV-specific CD4 T cells (a type of immune cell).

Introduction

Chronic HBV infection is a worldwide epidemic, affecting over 250 million people.[1] Patients typically require life-long therapy because nucleos(t)ide analogue treatment rarely induces sustained cure. Effective immunological control of HBV replication, *i.e.* functional cure, is observed in a small subset of chronically infected patients, mostly in the context of antiviral therapy.[2] By contrast, functional cure is the standard outcome following acute infection in adults. Therefore, restoration of HBV immune control through immunotherapeutic interventions appears to be an achievable objective.[3]

In both humans and animals, T cells are required for sustained control of HBV.[4–6] CD8 T cells are considered to mediate viral clearance, while CD4 T cells orchestrate effective CD8 T cell and B cell/antibody responses.[7] To date, the majority of immunological studies in HBV infection have focused on CD8 T cells, whereas data based on direct *ex vivo* analysis of HBV-specific CD4 T cells is extremely limited.[8,9] Overall, the detailed requirements for sustained control of HBV remain incompletely understood, and significant challenges remain for a better understanding of HBV immunity. One challenge is the complexity of the natural history of HBV infection that evolves over decades, hampering longitudinal studies aimed at dissecting the dynamics of immunological control and disease activity. Additionally, the frequencies of HBV-specific T cells in the blood are very low for CD8 T cells and even lower for CD4 T cell populations, and most T cell studies, including current work,[10] investigate HBV-specific T cells after

Keywords: Hepatitis B virus; functional cure; CD4 T cells; T cell function; HLA multimer.
Received 12 December 2021; received in revised form 12 May 2022; accepted 25 May 2022; available online 15 June 2022
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https://doi.org/10.1016/j.jhep.2022.05.041

in vitro expansion, which significantly alters T cell phenotypes and function.[11]

HBV-specific CD4 responses are especially understudied, with only responses targeting a single CD4 T cell epitope analyzed using major histocompatibility complex (MHC) class II multimers.[8,9] Historical studies applying assays after *in vitro* expansion of specific T cells primarily described T helper type 1 HBV-specific CD4 responses, mostly targeting core protein, and to a lesser degree envelope, polymerase, and X proteins.[12–16] To understand the role of CD4 immunity in HBV infection, we comprehensively mapped HBV-specific T cell responses using interferon-γ (IFN-γ) ELISpot in a large cohort of individuals with HBV; we also performed functional direct *ex vivo* analysis by intracellular cytokine staining (ICS) and direct *ex vivo* peptide–MHC class II multimer studies. Our data indicate that higher frequencies of functional HBV-specific CD4 T cell responses are a key component of the immune signature associated with functional cure, and that direct ex *vivo* analysis of HBV-specific CD4 T cells, as demonstrated here, is a promising approach for identifying the molecular correlates of CD4-mediated immune protection.

Materials and methods

HBV cohort

We studied a total of 124 patients infected with HBV who were recruited at Massachusetts General Hospital, Boston, USA; at Fiocruz Hospital, Rio de Janeiro, Brazil; and by Janssen Pharmaceuticals (Immune carta cohort), Beerse, Belgium. Participants included patients following an adult acute infection (n = 59), patients with chronic HBsAg-positive infection (n = 54), and patients with functional cure of chronic infection (*i.e.* HBsAg seroconversion [n = 11]). The following HBV diagnostic criteria were used: Acute resolving HBV infection was defined as the development of acute hepatitis, with HBsAg and anti-HBc immunoglobulin-M (anti-HBc-IgM) positivity. Chronic HBsAg-positive patients were identified by HBsAg (>6 months) and anti-HBc IgG positivity. Chronic HBsAg-negative patients were defined by undetectable HBV DNA and HBsAg with or without anti-HBs seroconversion (detectable anti-HBs (9/11), data not available (2/11)).[17] All participants tested negative for HIV. The respective local institutional review boards gave approval for this study, and all participants provided informed consent.

Matrix IFN-γ ELISpot assays

IFN-γ ELISpot assays were performed as previously described[18] and set out in the supplementary methods.

In vitro generation of peptide-specific HBV T cell lines

Peripheral blood mononuclear cell (PBMC) samples (5×10^6 to 10×10^6 cells at 1×10^6 cells/ml) were stimulated with single overlapping HBV peptides (10 μg/ml) for 14 days in R10/50 medium (R10 containing recombinant interleukin-2 [IL-2; 50 U/ml; Sigma-Aldrich]). Every other day, fresh R10/50 medium was added.

Intracellular cytokine staining

For direct *ex vivo* ICS, 60 to 80 million PBMCs were seeded at 1×10^6 cells per ml of R10 medium and rested overnight at 37°C. For acute infection, 20 million PBMCs were used. Samples were stimulated with pools of single overlapping peptides (OLPs) representing the HBV core (n = 2 pools) and envelope (n = 3 pools) proteins at 10 μg/ml protein for 6 h; a protein transport inhibitor was added after 2 h. Intracellular protein staining was performed for IFN-γ, tumor necrosis factor-α (TNF-α), and IL-2. Responses were considered positive if the stimulated signal was twice the unstimulated signal with at least 10 HBV-specific T cells. In order to increase specificity of the assay, functional HBV-specific T cells were identified based on cytokine production of cells that also upregulated CD154 (for CD4 T cells[19]) or CD69 (for CD8 T cells), respectively. The negative condition was used to subtract any background staining and the cumulative percentage of cytokine-producing cells was calculated. The slightly modified ICS assay for the T cell lines is described in the supplementary methods.

MHC class II multimers

MHC multimers were obtained from the National Institute of Health (NIH) Tetramer Core Facility (Atlanta, USA) and the Benaroya Research Institute Tetramer Core Laboratory (Seattle, USA). HLA class II multimers were synthesized for 6 distinct epitopes restricted by 4 different HLA alleles (Table S2). Multimers were labeled either with phycoerythrin or allophycocyanin.

Flow cytometry and multimer staining

Cell samples were washed with PBS, pelleted, and incubated with a viability dye (Invitrogen LIVE/DEAD Fixable Blue) for 30 min on ice. Cells were washed and pelleted. Multimers were then applied for 1 h at 37°C, resuspended every 20 min, washed, and pelleted. HBV multimer-positive cells were enriched using MACS columns (Miltenyi Biotec), according to the manufacturer's protocol. Cell samples were then stained with surface antibodies (Table S2) for 30 min on ice and either resuspended in 2% paraformaldehyde (Fisher Scientific) in PBS or stained for intracellular proteins. Intracellular proteins were stained using the FOXP3 fixation/permeabilization buffer kit (BioLegend). Compensation and rainbow beads (BD Bioscience, 552843 and 556298, respectively) were used for compensation and for laser calibration. Cell samples were acquired on a Becton Dickinson LSR 2 flow cytometer.

Statistical analysis

Data analysis was performed using FlowJo LCC version 10 and GraphPad Prism software version 9.2. The Wilcoxon paired test and the Mann-Whitney *U* test were used for statistical analysis. For correlations, a Spearman rank correlation coefficient was calculated. Data were summarized using the median and interquartile range (IQR). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ and $^{****}p \leq 0.0001$.

Results

Using PBMCs from a total of 118 participants, we performed IFN-γ ELISpot, ICS, and MHC multimer assays to determine HBV-specific T cell responses directly *ex vivo*. Participants were divided into 3 different clinical groups: 1) adult acute HBV infection resulting in cure in all but one case, 2) chronic HBsAg-positive infection, and 3) chronic HBV infection after HBsAg seroconversion. The clinical characteristics of the HBV cohort with the individuals studied in each of the different assays are summarized in Table S1.

Acute HBV infection is characterized by strong and broadly directed T cell responses

We first mapped HBV-specific T cell responses using PBMCs and 198 overlapping peptides covering all HBV proteins in an IFN-γ ELISpot. PBMCs were studied directly *ex vivo*, without prior *in vitro* expansion. In total, we studied 53 individuals, 27 following an acute resolving infection, 16 with chronic HBsAg-positive infection, and 10 individuals with chronic HBsAg-negative infection. Acute patients were studied at a median time of 8.5 weeks (IQR 6–12 weeks) after onset of symptoms. Using the approach outlined in Fig. S1A–F, we detected HBV-specific T cells in 24/27 (89%) of the acute patients *vs.* 9/16 (56%) in chronic HBsAg-positive infection, and 8/10 (80%) in chronic HBsAg-negative individuals. Overall, HBV-specific T cell frequencies were significantly higher in acute infection (median 423 cumulative spot-forming units (SFUs) per million cells, IQR

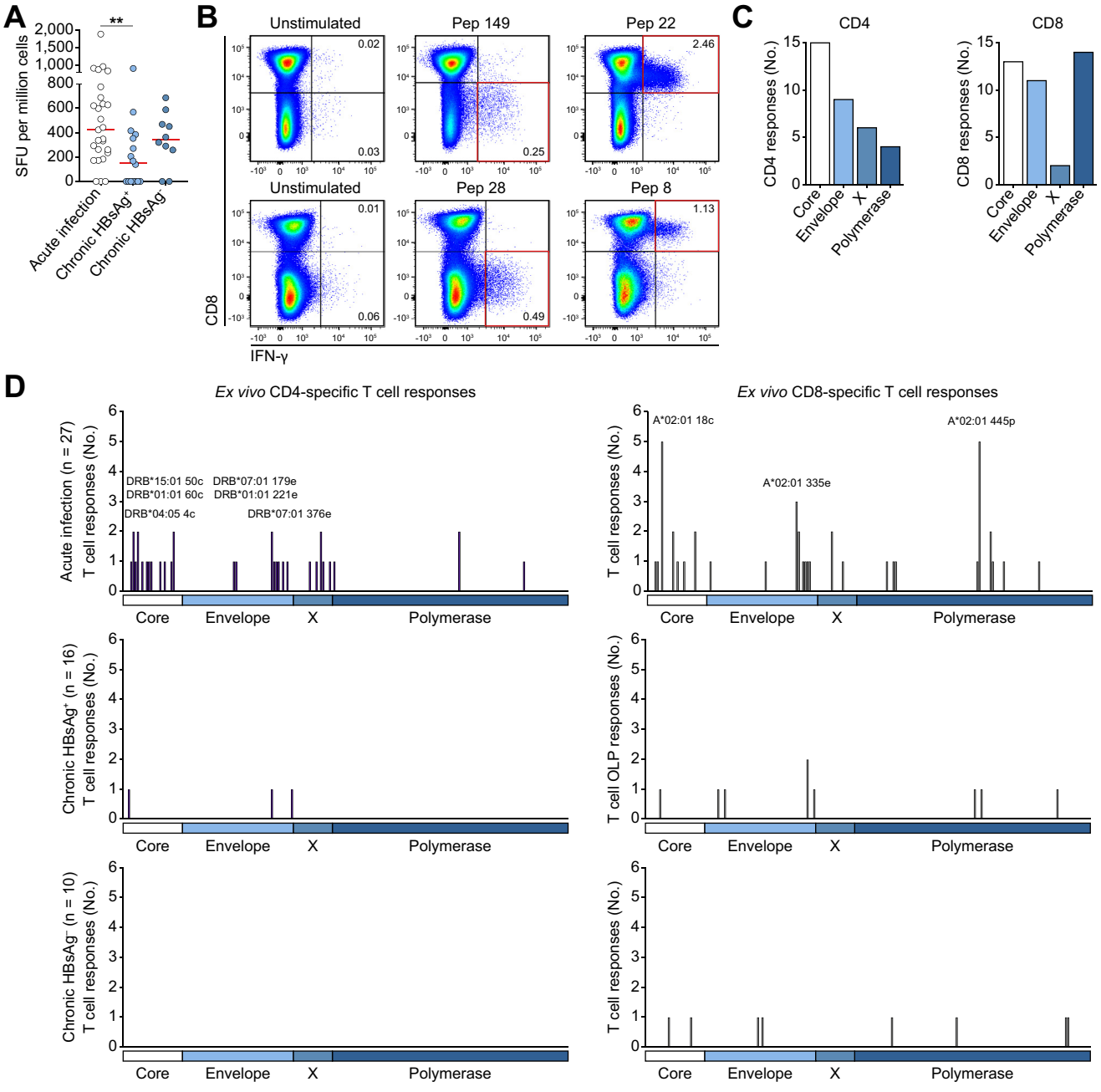

Fig. 1. The breadth and location of HBV-specific CD4 and CD8 T cell responses. (A) Total number of SFUs per million for each patient (Mann-Whitney *U* test; $^{**}p \leq 0.01$). (B) Example of intracellular cytokine staining of peptide-specific T cell lines. The percentage of IFN-γ-producing cells is shown for the unstimulated and single peptide-stimulated conditions. First sample (top) was studied 5 weeks after the onset of symptoms, second sample (bottom) was studied at week 14. (C) The number of CD4 and CD8 T cell responses targeting different HBV proteins is displayed for patients with acute resolving HBV. (D) Relative genomic location of confirmed CD4- and CD8-specific T cell responses in patients with an acute infection (top), chronic HBsAg-positive infection (middle), and chronic HBsAg-negative infection (bottom). Pep, peptide; SFUs, spot-forming units. (This figure appears in color on the web.)

248-633) compared to chronic HBsAg-positive patients (median 154 SFU/Mio, IQR 0-360, p <0.005) and to a lesser degree HBsAg-negative individuals (median 341 SFU/Mio, IQR 265-463, p = 0.055) (Fig. 1A). A similar picture emerged for the breadth of the response, with acute cases exhibiting a median of 5 distinct OLP pool responses (IQR of 2–10) *vs.* a median of 2 OLP responses in both subgroups of chronically infected patients (IQR 0-5 for HBsAg-positive and IQR 2-5 for HBsAg-negative patients) (Fig. S1G,H). We further differentiated between CD4 and CD8 specificities using T cell lines and ICS (Fig. 1B), successfully confirming 37 distinct CD8 specificities and 30 CD4 specificities out of 94 single peptide responses (Table S3). In patients with acute self-limiting infection, we observed a broad pattern of recognition covering all HBV proteins, with single peptide responses targeting core protein (n = 28), envelope (n = 20), polymerase (n = 18), and X (n = 8) proteins (Fig. 1C,D). While both core and envelope proteins were equally targeted by CD4 and CD8 T cell responses, the polymerase protein was targeted more frequently by CD8 T cells and X protein by CD4 T cells. In individuals with chronic infection (both HBsAg-positive and HBsAg-negative), we detected almost exclusively HBV-specific CD8 T cell responses targeting different HBV proteins without a clear dominance. In sum, the *ex vivo* ELISpot data showed strong and broadly directed T cell reactivity in acute self-limited HBV infection, confirmed a diminished HBV-specific T cell response in patients with chronic HBV infection (with an even weaker CD4 response compared to that of CD8 T cells) and identified novel HBV T cell epitopes, especially those targeted by CD4 T cells.

Functional HBV-specific CD4 T cell responses are associated with HBV control

Given the low detection rate of HBV-specific T cell responses in the ELISpot assay, especially in chronic patients, we decided to utilize direct *ex vivo* ICS after stimulation with HBV core and envelope peptides to investigate the frequency of functional HBV-specific T cell responses. Using at least 60 million PBMCs per assay allowed for increased sensitivity beyond that of the ELISpot, which cannot easily be performed using more than 200,000 cells per condition due to rapidly increasing background signal. In addition, the ICS also allows for the parallel assessment of CD4- and CD8-mediated T cell responses. We studied IFN-γ, TNF-α and IL-2 production in 5 acute, 6 chronic HBsAg-positive, and 8 chronic HBsAg-negative patients (Fig. 2A and Fig. S2A-D). Clinical characteristics for these patients are displayed in Table S1. Acute patients were studied at a median time of 3 weeks after the onset of symptoms. For the chronic HBsAg-positive patients we selected those with undetectable or very low viral loads (all but one on treatment), as they have the highest likelihood of functional T cells.[20] We detected at least one CD4 or CD8 IFN-γ-producing T cell response against core or envelope in 18/19 (95%) patients compared with 29/53 (55%) in the ELISpot. Similar to the ELISpot, frequencies of functional cells were highest after control of acute infection, but significantly greater numbers of functional CD4 T cells compared to chronic HBsAg-positive patients were also observed in chronic HBsAg-negative patients (Fig. 2B). These response patterns were similar for all 3 cytokines tested (IFN-γ, TNF-α, and IL-2), with similar results for core and envelope responses (Fig. S2A,B). Both groups of functional cure patients were also more likely to have both CD4 and CD8 T cell responses simultaneously (Fig. S2C). Multifunctionality of specific T cells was consistent within response types when it could be assessed (Fig. S2D). In contrast, frequencies of HBV-specific CD8 T cells alone did not show similarly clear differences between the different patient groups (Fig. 2C). These results suggest that functional HBV-specific CD4 T cell responses have a stronger correlation with functional cure than their CD8 counterparts.

The presence of HBV-specific tetramer+ CD4 T cells is a hallmark of HBV functional cure

We next wanted to test whether the difference in CD4 responsiveness was reflective of differences in specific T cell frequencies or mostly based on distinct functionality of HBV-specific CD4 T cells. To this end, we utilized the results from the ELISpot mapping to synthesize a library of 6 class II HLA tetramers that enabled the enumeration of HBV-specific CD4 T cells independently of function and the phenotyping of these cells directly *ex vivo* (Fig. 3 and Fig. S3). We screened a total of 113 individuals expressing at least one of the alleles for which HBV tetramers were available. Clinical characteristics for the HBV patients are displayed in Table S1. In the group with acute infection, 33/39 (85%) patients had detectable HBV-specific CD4 T cells (median 0.0075% of total CD4), with some patients recognizing 2 specificities for a total of 37/51 (73%) positive tetramer assays (Fig. 3AD). In these post-acute infection patients, HBV-specific CD4 T cells were often detectable without the need to increase the sensitivity of the assay by magnetic bead enrichment of tetramer populations. In contrast, the more sensitive enrichment approach was always required in chronic cases, with HBV-specific CD4 tetramer populations much more readily observed in the patients with functional cure. In this group, 5/6 (83%) patients had a response, with 9/10 (90%) assays positive compared with chronic HBV infection where only 7/41 (17%) patients and 7/58 (12%) tetramers tested positive. Interestingly, the few positive responses in chronic HBsAg-positive patients were observed over a wide range of HBV DNA levels, from undetectable to more than 5×10^8 IU/ml. Specific CD4 T cells were equally rare in untreated, chronic HBsAg-positive patients with a low HBV viral load (<2,000 IU/ml: 2/13 [15%]), a high viral load (>2,000 IU/ml: 2/10 [20%]), and in treated patients (with treatment: 3/18 [17%] *vs.* no treatment 4/23: [17%]). We also tested the presence of envelope-specific responses in healthy HBV-vaccinated individuals, who had a response rate of 15/27 (56%). These results confirm that protective immunity against HBV is associated with the more frequent physical presence of HBV-specific CD4 T cell populations, not just with better functionality of these cells.

Acute HBV resolution is associated with the emergence of central memory CD4 T cells

Being able to characterize HBV-specific CD4 T cells directly *ex vivo*, we first defined CD4 T cell phenotypes in acute HBV infection longitudinally (at a median of 3 and 34 weeks after symptoms, respectively). The early time point reflects acute control of HBV whereas the later time point describes the CD4 profile during maintenance of functional cure. The individual positive HBV-specific CD4 tetramer responses are shown in Fig. S4, and samples of phenotypic analysis are displayed in Fig. 4A. During the early phase of infection, we observed predominantly effector memory CD4 T cells, characterized by low

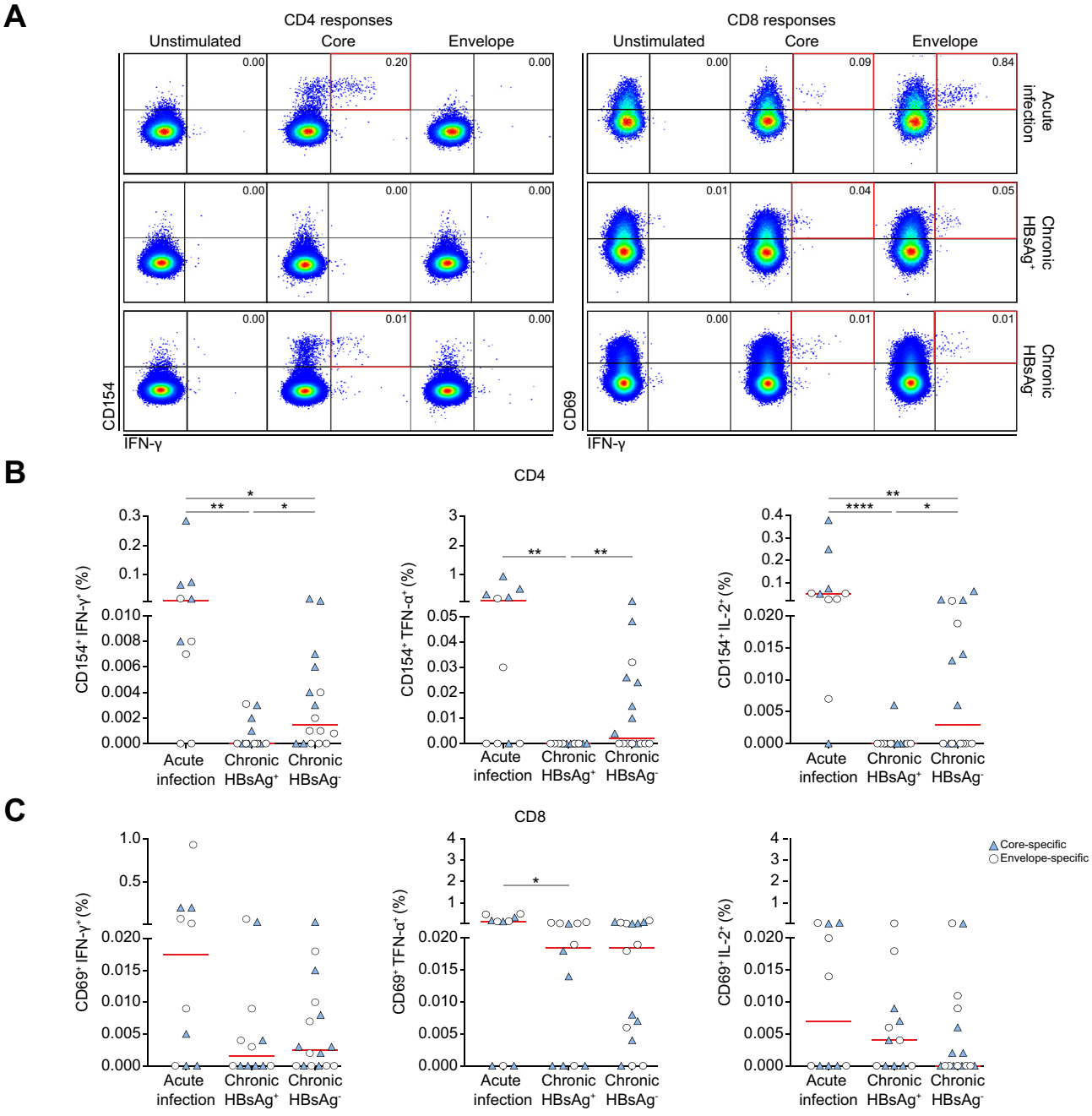

Fig. 2. HBV-specific T cell functionality in different stages of infection. (A) Detection of activated CD4 T cells ($CD4^+CD154^+$) and cytokine secretion in response to stimulation with the HBV core and envelope protein. Example staining of IFN-γ production from $CD4^+CD154^+$ cells in response to stimulation with single OLPs representing the core and envelope proteins. Results from one OLP pool are shown for an acute patient, a chronic HBsAg-positive patient, and an HBsAg-negative patient. Additional example stainings for TNF-α and IL-2 are displayed in Fig. S2. Frequencies of IFN-γ, TNF-α, and IL-2 production from $CD4^+CD154^+$ (B) and $CD8^+CD69^+$ (C) T cells are displayed. A red line indicates the median (Mann-Whitney *U* test; $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$). OLPs, overlapping peptides. (This figure appears in color on the web.)

expression of CD45RA, CCR7, and CD62L. At this time point, expression of the programmed cell death protein 1 (PD-1) was almost universal, whereas other molecules had rather heterogeneous expression patterns across the cohort. Expression of cytotoxic T-lymphocyte associated protein 4 (CTLA-4), inducible T cell costimulator (ICOS), T-bet (TBX21), and eomesodermin (Eomes) ranged between 0% to almost 100% of tetramer-positive CD4 T cells (Fig. 4A,B). On the lower end of the expression spectrum were Ki-67 and OX40, which we detected only in a subset of tetramer-positive cells in few patients. The complete longitudinal results are displayed in Fig. S5, showing both percentage of HBV-specific CD4 T cells and median fluorescence intensity. These data show the evolution of the CD4 response with viral control, with the phenotype evolving towards a mix of

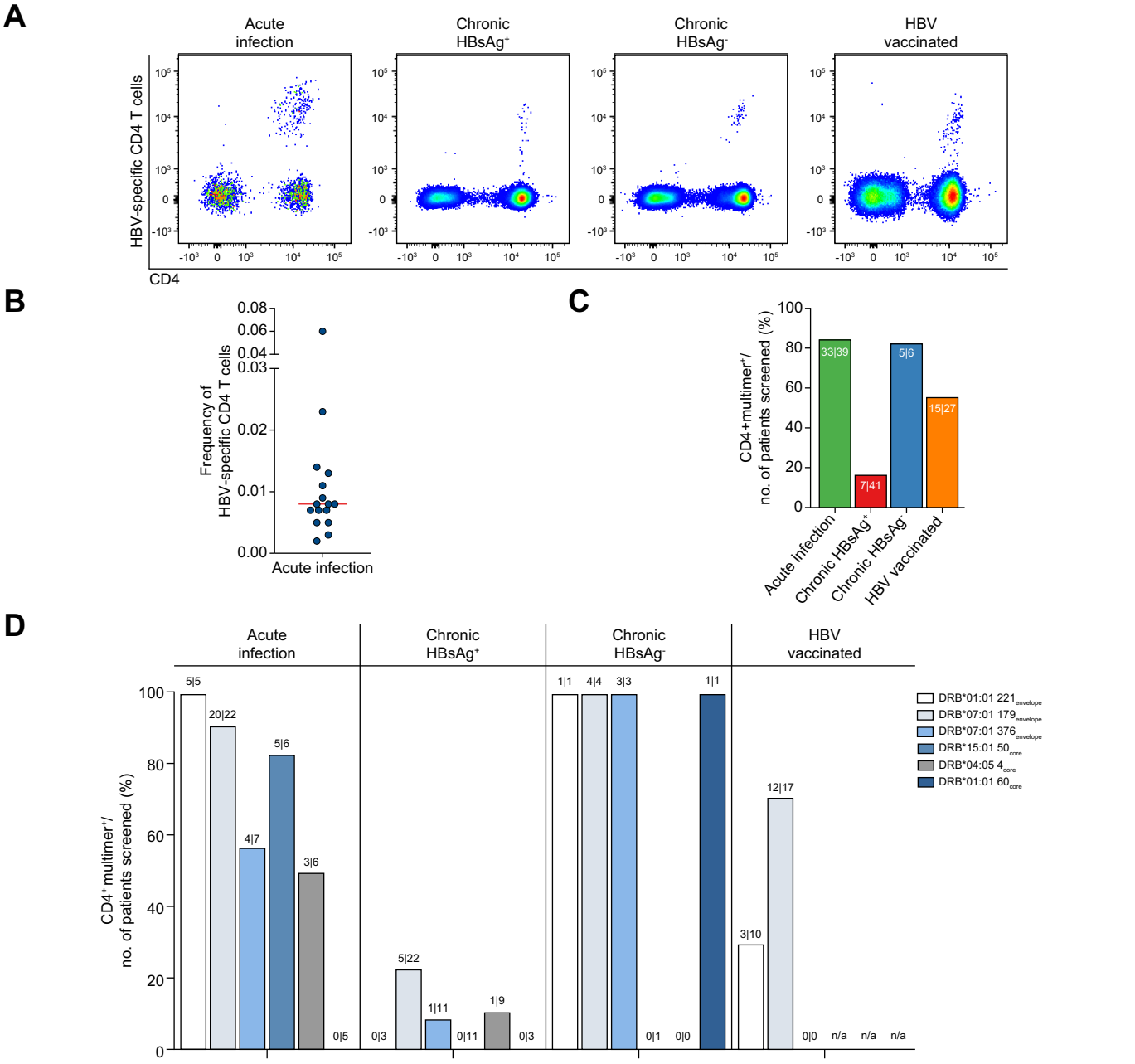

Fig. 3. Detection of HBV-specific CD4 T cells in different stages of infection. (A) Examples of *ex vivo* HBV-specific CD4 T cell responses in individuals with HBV at different stages of infection and healthy HBsAg-vaccinated individuals. All results in chronic patients are after magnetic bead enrichment. (B) Frequencies of HBV-specific CD4 T cells without magnetic bead enrichment are shown for 16 patients with acute HBV. (C) Summary of the CD4 multimer screening results. The number of patients with detectable HBV-specific CD4 T cells is indicated. (D) Summary of the CD4 multimer screening results shown for each epitope. (This figure appears in color on the web.)

central and effector memory and more homogenous expression of most other markers, as CD38, CTLA-4, ICOS, T-bet, Eomes, and Ki-67 were all downregulated to low expression levels. PD-1 expression also decreased in most patients, but significant expression of PD-1 was observed on many HBV-specific CD4 T cell populations. We were able to follow one adult patient with acute HBV who failed to control HBV and progressed to chronic infection (Fig. 4C). Interestingly, the phenotype of the HBV-specific CD4 T cells evolved comparably to what was observed for acute resolving infection for most of the markers studied, with the exception that the cells remained completely in the effector memory state. While this analysis is still relatively narrow, based on the requirement for large cell numbers in these assays and limited availability of patients that lose HBsAg, the data show evolution from highly activated effector CD4 T cells in early acute infection towards a resting CD4 phenotype with increasing features of central memory once functional cure is achieved.

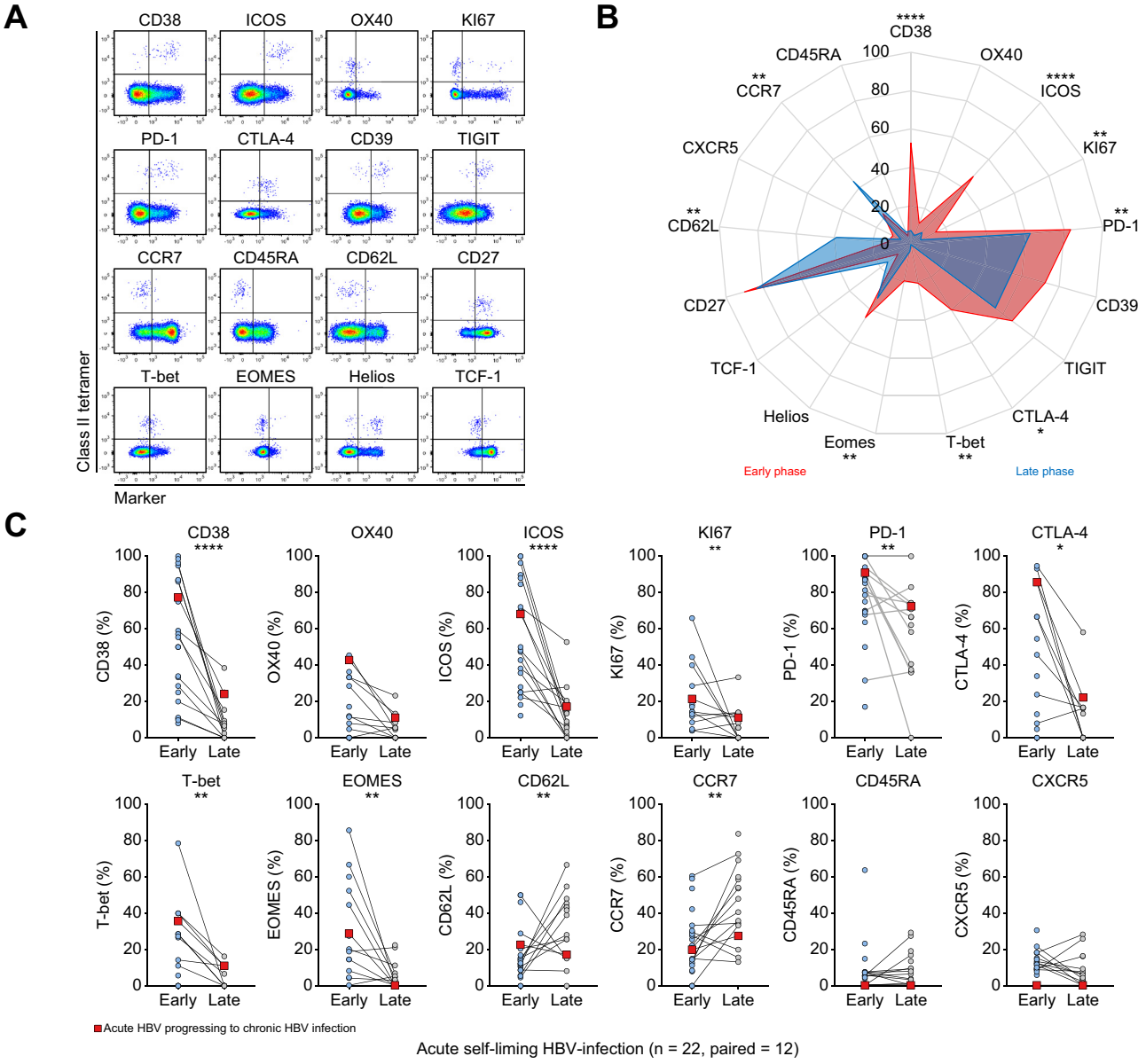

Fig. 4. Longitudinal analysis of the HBV-specific CD4 T cells following acute resolving infection. (A) Example of the immunophenotype of Env_{179s}-specific CD4 T cells in a patient following an acute HBV infection. (B) Radar plot depicting the median expression of each marker before (red) and after (blue) control of HBV infection. (C) Interindividual expression variability. The red square indicates one acute HBV case that progressed to a chronic HBV infection. (B–C: Wilcoxon paired test; $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$). (This figure appears in color on the web.)

HBV-specific CD4 central memory T cells are rare in chronic infection, but not in functional cure

Extending the phenotypic analysis of HBV-specific CD4 T cells to chronic HBV patient populations was even more challenging due to the even lower frequency of these cells in these patient populations and thus the need for large numbers of PBMCs. We therefore focused this part of the analysis on a characterization of the memory phenotype, T cell activation, and inhibitory receptor expression on HBV-specific CD4 T cells. Like functional cure after adult acute infection, we found HBV-specific CD4 T cells with both effector and central memory phenotypes in chronic HBsAg-negative patients as well as HBV-vaccinated healthy individuals (Fig. 5A). By contrast, chronic HBsAg-positive infection was characterized by a complete dominance of effector memory CD4 T cells, similar to what we had observed during the viremic phase of acute infection (Fig. 5B). Expression of PD-1 was highly variable, with only CD4 responses primed by HBV vaccination displaying consistently low PD-1 expression (Fig. 5C). Overall, the data show the presence of central memory type CD4 T cells as a unifying feature of protective HBV-specific CD4 T cell responses in functional cure both after acute and chronic HBV infection.

Discussion

Functional cure of HBV infection is considered an achievable therapeutic goal, given that it is almost universally observed after adult infection and, in a minority of patients, even after many years of chronic HBV infection.[21] However, the design of immunotherapeutic interventions able to induce effective and lasting HBV control has been hampered by a limited

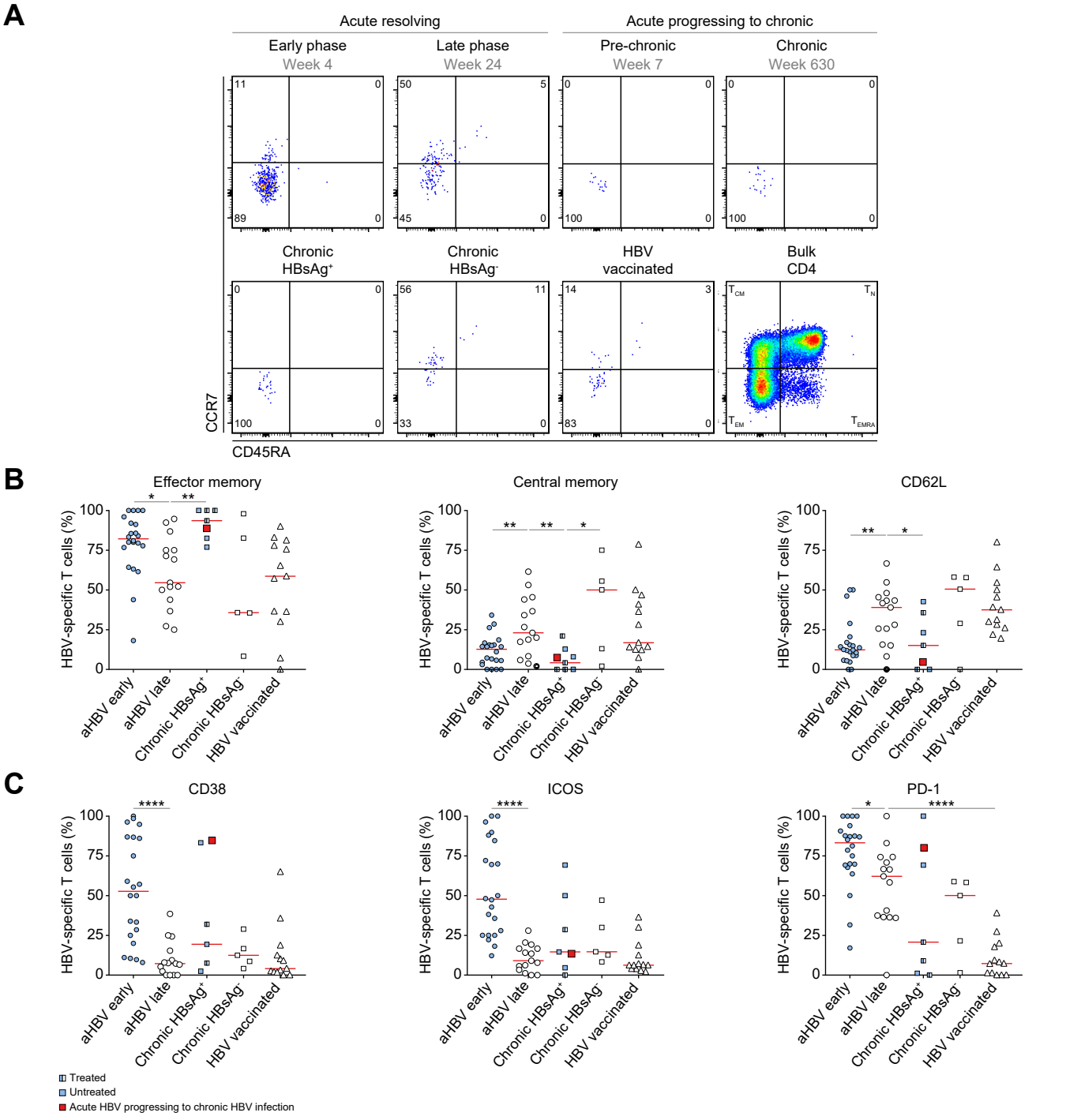

Fig. 5. HBV-specific CD4 T cell phenotype and memory development in chronic infection. (A) Coexpression analysis of CD45RA and CCR7 to determine T cell differentiation state on total CD4 and HBV-specific CD4 T cells. The frequencies of central memory (CCR7-positive/CD45RA-negative), naive (TN = CCR7/CD45RA double positive), effector memory re-expressing CD45RA (CCR7-negative/CD45RA-positive), and effector memory (CCR7/CD45RA double negative) populations are shown. (B) Cross-sectional analysis of the HBV-specific CD4 T cell memory phenotype in distinct HBV patient populations and in healthy HBsAg-vaccinated individuals. (C) Cross-sectional analysis of the HBV-specific CD4 T cell activation phenotype in distinct HBV patient populations and in healthy HBsAg-vaccinated individuals. Red line indicates the median. (B–C: Mann-Whitney *U* test; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, **** $p \leq 0.0001$). (This figure appears in color on the web.)

understanding of the HBV-specific T cell responses that are required to control HBV. Currently, we do not know the molecular characteristics of effective HBV immunity by either CD4 or CD8 T cells in detail, as the extremely low frequencies of HBV-specific CD8 and especially CD4 T cells have impeded direct *ex vivo* analyses.[3,22,23] It also remains an open question whether T cells that control HBV after acute adult infection target different epitopes or are molecularly distinct from those after HBsAg loss following chronic infection, and, thus, whether different immunological pathways regulate can lead to functional cure. Here, we

studied patients with acute self-limiting HBV infection as well as those in different stages of chronic infection using 3 different direct *ex vivo* T cell assays. We focused on the CD4 response due to the extreme paucity of published studies analyzing HBV-specific CD4 T cells in detail directly *ex vivo*.[8,9]

The most important conclusion from our study is that more readily detectable functional HBV-specific CD4 T cell responses are associated with persistent HBV control, both after self-limiting acute infection and following chronic infection. This conclusion is supported by data from 2 independent direct *ex vivo* CD4 assays, one relying on T cell function and the other on physical detection via T cell receptor binding. Importantly, both assays found significant differences in CD4 response detection despite the relatively small numbers of patients with functional cure after chronic HBV infection (who are difficult to recruit). In the functional assay assessing cytokine production after stimulation with core and envelope peptides, we were able to directly compare the results for CD4 T cells with those restricted by CD8 T cells. While HBV-specific CD8 T cell numbers were generally higher than those of CD4 T cells, their frequencies did not associate as clearly with HBV control. This does not exclude a role for CD8 responses in HBV control, as the study may be underpowered to detect more subtle differences. In contrast, the highly significant differences for CD4 T cells in the same assay support a renewed focus on investigating the T helper response in HBV infection and suggest CD4 T cells as potential candidates for immunotherapeutic agents.[24] Given the established roles of CD4 immunity for both effective CD8 T cell and B cell responses in virtually all viral infections,[7] they should be included in any comprehensive HBV immune analysis or immunotherapy monitoring, despite the technical challenges.

The CD4 tetramer data reveal additional insights beyond confirming higher CD4 T cell frequencies in functional cure. Regarding the targeting of different HBV proteins, we found just as many positive multimer responses to envelope proteins as to core proteins across all disease stages and after HBV vaccination. This is distinct from the known CD8 multimer responsiveness, where in chronic infection, but to a lesser degree also in acute infection, core protein-specific responses are much more readily detected, at least in HLA-A2 patients.[22] As the core response by ICS (assessing entire proteins) was, in most cases, stronger than that targeting the envelope protein, it seems possible that this observation is somewhat biased by our current HLA class II multimer set. It is also noteworthy that the HBV genotype might influence the response to specific CD4 epitopes, as has been shown for CD8 T cells.[25] The previous HBV CD4 studies were mostly performed in chronic genotype D infection,[8] whereas, in this study, genotype D was rare in the acute patients from Brazil.[26] In any case, the reliable detection of envelope CD4 responses in functional cure and in patients with vaccine-induced HBV protection opens an important avenue for further investigation.

Based on the higher frequency of HBV-specific CD4 T cells in acute infection, we could perform a relatively broad longitudinal phenotyping analysis in patients during acute infection and subsequent extended control of HBV replication. During the viremic phase, we found highly activated effector memory T cells expressing PD-1, CTLA-4, and ICOS, together with partial expression of the transcription factors T-bet and Eomes. A substantial number of the HBV-specific cells also expressed Ki-67 as a signature of active proliferation. We previously described similar CD4 populations in acute HCV infection, irrespective of outcome, supporting the hypothesis that high expression of inhibitory molecules is a physiological response to allow regulation of the CD4 response.[27] Indeed, this upregulation is temporary in adult acute HBV infection as well as in acute self-limited HCV infection, in contrast to patients developing persistent HCV viremia.[27] Interestingly, the downregulation of CTLA-4 is in both infections faster and more complete than that of PD-1, fully normalizing shortly after viral control is achieved, further highlighting that PD-1 expression *per se* is not necessarily linked to T cells being ineffective. At the same time, most cells reach a steady state with low activation and proliferation, loss of detectable expression of the transcription factors Eomes and T-bet, and the emergence of a central memory T cell phenotype.

In chronically infected patients we could not assess as many markers as in the acute patients, because of the even larger number of cells required for tetramer staining combined with magnetic bead enrichment, as well as the limited access to chronic patients that have lost HBsAg. We found that chronic patients with functional cure mostly share the same mixed phenotype of effector and central memory phenotypes as observed in functional cure after adult acute infection and after vaccination of healthy individuals. This was distinct from HBsAg-positive chronic infection, where almost all cells were effector memory cells, as in the viremic phase of acute infection. Despite this phenotype, cells in chronic infection were not universally more activated and did not always express higher levels of PD-1 than either group with functional cure, with all groups showing PD-1 expression over a wide range. Even HBV vaccination did not result in completely uniform HBV-specific CD4 T cells. To what degree these mixed phenotypes are a consequence of differences between T cell responses targeting different HBV proteins, as we and others have demonstrated for CD8 T cells, remains to be determined.[11,28,29] Overall, our study indicates significant similarities for the CD4 T cell response between functional cure after acute and after chronic HBV infection, for both the targeted HBV epitopes and the T cell differentiation state. More definite answers about the state of the CD4 response in patients with chronic HBV and the CD4 profiles that should be induced by targeted immunotherapies will require studies utilizing an even greater library of class II reagents together with larger blood donations (*e.g.*, through leukapheresis) and with the use of RNAseq and other 'omics' approaches that can simultaneously assess more molecules.

There are several important technical conclusions to be drawn from our data that can inform future studies on HBV-specific CD4 responses. First, the ELISpot assay clearly has significant limitations in the analysis of HBV-specific T cell responses, especially in the chronic phase of infection and even more for CD4 T cells compared with CD8 T cells. Refinement of this assay (*e.g.*, the Fluorospot) can improve its sensitivity, but there are limits given the maximum number of input cells per well.[30] In this study, the main contribution from the ELISpot screening was the mapping of novel HBV epitopes, mostly in the acute infection setting, that is essential for expanding the HBV T cell analysis toolbox. Based on our data thus far (including unpublished CD8 tetramer data), epitopes identified in acute infection are also targeted in chronic infection and thus will be useful across the spectrum of HBV infection. Second, more comprehensive studies of the CD4 T cell response will not only require an advanced toolbox of class II multimers; but, given the

large number of cells required for direct *ex vivo* CD4 T cell assays, studies and trials should ideally be designed with larger blood draws than routinely obtained, at least for a subgroup of patients. As larger blood draws are not easily integrated into clinical trials an alternative might be the generation of large libraries of barcoded class II tetramers that would allow for a broader simultaneous detection of multiple HBV-specific populations, thus reducing the need for more PBMCs.[31] Finally, the CD4 multimer approach should also be used to study intrahepatic immune cells. Whether HBV-specific CD4 T cells are compartmentalized to the liver like their CD8 counterparts is unknown and it is quite possible that CD4 T cells, as indirect mediators of immunity, reside in local lymph nodes rather than in the liver.

In summary, we have utilized direct *ex vivo* assays based on T cell function as well as physical detection to assess the HBV-specific CD4 T cell response in functional cure after both acute and chronic HBV infection. That both assays detect significantly more and larger responses in cured compared to chronic patients supports a critical role of the HBV-specific CD4 T cell responses in viral control and suggests that immunotherapeutic approaches for chronic HBV infection should also target HBV-specific CD4 cell immunity, not just CD8 responses. Our results further demonstrate the feasibility of performing direct *ex vivo* CD4 T cell assays that can aid in the rational design and monitoring of immunotherapeutic interventions intended to revive the CD4 T cell response. As in most other viral infections, CD4 T cell function should not be overlooked as a cornerstone of viral control.

Abbreviations

CTLA-4, cytotoxic T-lymphocyte associated protein 4; Eomes, eomesodermin; HLA, human leukocyte antigen; ICOS, inducible T cell costimulatory; ICS, intracellular cytokine staining; IL-2, interleukin-2; IFN-γ, interferon-γ; MHC, major histocompatibility complex; OLP, overlapping peptide; PBMC, peripheral blood mononuclear cells; PD-1, programmed cell death protein 1; PMA, phorbol myristate acetate; SFUs, spot-forming units; TCR, T cell receptor; TNF-α, tumor necrosis factor-α.

Financial support

The work was funded by grant support from NIH (R01 AI148648 to G.M.L. and U19 AI082630 to G.M.L. and A.Y.K.), Janssen Pharma (to G.M.L.), NVGE Gastrostart 2019.25 (to R.C.H.), Deutsche Forschungsgemeinschaft (BA7175/1-1 to L.M.B. and DR1161/1-1 to H.D.).

Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

RH designed and performed experiments, analyzed the data, made the figures, and wrote the paper. SD performed experiments, analyzed the data, and made figures. LB, HD, MR, JC, and JT performed experiments and analyzed the data. JA, AG, PS, LX, LP, AP, FEB, AK, and LL contributed to the design and recruitment of the clinical cohorts and to data interpretation. AB contributed to data interpretation and revised the manuscript. GL conceived and supervised the study, analyzed the data, provided funding, and wrote the paper.

Data availability statement

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgements

We would like to acknowledge the patients and physicians involved in this study. The NIH tetramer facility for providing the HBV-specific multimers. The members of the Ambulatório/Laboratório de Hepatites Virais, Instituto Oswaldo Cruz/FIOCRUZ, Rio de Janeiro, Brazil: Vinicius da Motta de Mello, Barbara Vieira Lago, and Fransisco Campello do Amaral Mello. The graphical abstract was created with BioRender.com.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2022.05.041.

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